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Functional complexes of mitochondria with Ca,MgATPases of myofibrils and sarcoplasmic reticulum in muscle cells

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Abstract

Regulation of mitochondrial respiration in situ in the muscle cells was studied by using fully permeabilized muscle fibers and cardiomyocytes. The results show that the kinetics of regulation of mitochondrial respiration in situ by exogenous ADP are very different from the kinetics of its regulation by endogenous ADP. In cardiac and m. soleus fibers apparent K_m for exogenous ADP in regulation of respiration was equal to 300–400 μM. However, when ADP production was initiated by intracellular ATPase reactions, the ADP concentration in the medium leveled off at about 40 μM when about 70% of maximal rate of respiration was achieved. Respiration rate maintained by intracellular ATPases was suppressed about 20–30% during exogenous trapping of ADP with excess pyruvate kinase (PK, 20 IU/ml) and phosphoenolpyruvate (PEP, 5 mM). ADP flux via the external PK+PEP system was decreased by half by activation of mitochondrial oxidative phosphorylation. Creatine (20 mM) further activated the respiration in the presence of PK+PEP. It is concluded that in oxidative muscle cells mitochondria behave as if they were incorporated into functional complexes with adjacent ADP producing systems – with the MgATPases in myofibrils and Ca,MgATPases of sarcoplasmic reticulum.

Keywords: Muscle; Respiration; Mitochondrion; ADP; ATPase

1. Introduction

Regulation of mitochondrial respiration and oxidative phosphorylation in vitro, in isolated mitochondria, is well described since the classical works of Chance [1] and explained in molecular terms in the framework of chemiosmotic theory of Mitchell [2]. The oxidative phosphorylation itself – the ATP synthesis in mitochondria – is explained by a rotary mechanism discovered by Boyer and Walker's groups [3,4]. These fundamental mechanisms operate, without any doubt, also in mitochondria in vivo, where these organelles are integrated into cellular metabolic systems. However, understanding cellular mechanisms of regulation of mitochondrial activity in vivo is far from complete: it is not known which cytoplasmic factors are responsible for the feedback signaling from ATPases in myofibrils and in cellular

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and subcellular membranes to mitochondria and thus responsible for precise matching of the free energy production to its demand. Indeed, in the heart cells the workload and respiration rate may be increased by more than an order of magnitude at practically constant levels of phosphocreatine, creatine and ATP [5,6]. If one assumes the creatine kinase equilibrium, the calculated ADP content will also be constant, in obvious conflict with the respiratory control concept of Chance. The alternative explanation that respiration rate may be regulated by calcium ions in parallel with regulation of contraction may be questioned, since inhibition of calcium uptake does not change the relationship between workload and oxygen uptake [7,8].

The difficulties of interpretation of these in vivo experimental data were most possibly caused by two reasons: first, an assumption of the creatine kinase equilibrium seems to be an oversimplification [9], and second, the phenomena of subcellular compartmentation and specific organization of the cell interior were not accounted for. For several years, we have studied these problems by using the permeabilized cell technique, which allows us direct access to different subcellular compartments in the cell in situ [10–15]. These studies have shown that the mitochondrial apparent affinity for exogenous ADP is much lower in the cells in situ than in isolated mitochondria in vitro [10–12]. This is most possibly due to the influence of intracellular structures on the function of mitochondrial outer membrane in the cells [10–14].

In the current study we develop this direction of research further and show that the kinetics of regulation of mitochondrial respiration in situ, under conditions of their natural interaction with other intracellular structures, depend on the source of ADP. These results are taken to show the importance of intracellular organization of energy metabolism for feedback signaling and the presence of serious restrictions for free diffusion of ADP in the cells.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 300–350 g and wild type C57BL/6 mice were used in experiments. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication no. 85-23, revised 1985).

2.2. Preparation of skinned muscle fibers

Skinned fibers were prepared according to the method described earlier [15]. The animals were anaesthetized with sodium pentobarbital (50 mg/kg body weight, intraperitoneal), chest opened and hearts when still beating excised and put into cooled solution A. Cooled hearts were cut into halves and muscle strips (3–5 mm long and 1–1.5 mm in diameter, 5–10 mg of wet weight) cut from endocardium of left ventricles along fiber orientation to avoid mechanical damage of the cells. Muscle fiber bundles (3–4 mm long, about 1 mm in diameter) were taken also from m. soleus (oxidative, slow twitch) and m. gastrocnemius white (glycolytic, fast twitch). By using sharp-ended forceps or needles, the muscle fibers were separated from each other leaving only small areas of contact. After that the fibers were transferred into vessels with cooled (in ice) solution A containing 50 μg of saponin per ml and incubated at mild stirring for 30 min for complete solubilization of the sarcolemma. Permeabilized (skinned) fibers were then washed in solution B for 10 min; this procedure of washing was repeated two more times to remove completely all metabolites, especially trace amounts of ADP. Complete removal of ADP can easily be seen from respiration recordings which should show very reproducible initial state 2 rates (designated as v_0) not sensitive to inhibition by atractyloside (see below).

Isolation and culturing of adult cardiac myocytes was carried out as described by Kay et al. [16].

Isolation of rat heart mitochondria was carried out as described previously [10].

2.3. Determination of the kinetics of respiration regulation by ADP in skinned fibers and cardiomyocytes

The rates of oxygen uptake were recorded by using the two-channel high resolution respirometer (Oroboros Oxygraph, Paar KG, Graz, Austria) or Yellow Spring Clark oxygen electrode in solution B, contain-

ing respiratory substrates (see below) and 2 mg/ml of bovine serum albumin (BSA). Determinations were carried out at 25°C, solubility of oxygen was taken as 215 nmol per ml [17].

Some control experiments were carried out in Ca^{2+}-free sucrose medium of the following composition: 240 mM sucrose, 20 mM HEPES, pH 7.1, 1 mM EGTA, 4 mM $MgCl_2$, 3 mM KH_2PO_4, 5 mM glutamate and 2 mM malate.

The volume of the assay medium was 2 ml in all experiments.

2.4. Determination of the flux of the ADP produced in ATPase reactions

The skinned fibers of rat heart were prepared as described above, except that the washing medium B contained 5 mg/ml BSA. The role of mitochondria in rephosphorylating the ADP produced in ATPase reactions was estimated as a decrease in the flux through the external system of ATP regeneration (phosphoenolpyruvate (PEP)–pyruvate kinase (PK)–lactate dehydrogenase (LDH)) after switching to operation of the mitochondrial oxidative phosphorylation. Approximately 3 mg skinned fibers were incubated in the spectrophotometric (Perkin-Elmer Lambda 900) cuvette containing a medium B complemented with 5 mg/ml BSA, 5 mM PEP, 20 IU/ml PK, 20 IU/ml LDH and 0.24 mM NADH at 25°C. The medium was continuously stirred by using the magnetical stirrer operated by the Variomag® Telemodul (H+P Labortechnic GmbH, Germany). The changes in optical density at 340 nm were measured before and after addition of 1 mM ATP as well as after subsequent additions of the substrates (10 mM glutamate and 2 mM malate) and 98 μM atractyloside. The reaction rate was estimated from the stable and linearly time-dependent portions of recordings. Care was taken that neither of the coupled enzyme system components was limiting the rate of ATPase reaction. The total volume of the reaction mixture was 1.5 ml.

2.5. Determination of ADP concentration in the reaction medium

The oxygraphic samples were used immediately for the high performance liquid chromatography (HPLC) studies or frozen in liquid nitrogen and stored at −80°C until ADP concentration determination for not longer than 2 weeks.

2.5.1. Perchloric acid extraction

Perchloric acid 60% (100 μl) was added to oxygraphic samples (600 μl) in order to precipitate all proteins (BSA) present in the respiratory medium. Then, the samples were centrifuged at $17\,000\times g$ for 10 min (4°C). The supernatants (600 μl) were neutralized by a solution of 2 M $KHCO_3$ (420–470 μl depending on the samples). The samples were spun once more at $17\,000\times g$ for 10 min (4°C) and the supernatants loaded directly onto HPLC system. These neutralized extracts can be stored during a few weeks at −20°C without degradation of adenine nucleotides.

2.5.2. Chromatography

Separation and determination of adenine nucleotides in chromatographic samples were performed by HPLC according to two procedures at a standard 1 ml/min rate making use of the spectrophotometric detector at 253 nm.

1. The studies were performed using FPLC equipment and PepRPC HR 5/5 column from Pharmacia. The column was equilibrated with 50 mM KH_2PO_4 buffer containing 5 mM tetrabutylammonium hydrogen sulfate adjusted to pH 6.0 (eluent A). After the sample application the column was eluted by a linear 0–70% gradient of the initial eluent A supplemented with 40% of methanol (eluent B) for 14 min. Then the gradient was increased to 100% of eluent B for 1 min and maintained another 3 min to clean the column. For the return to eluent A, a decreasing 100–0% eluent B was used for 2 min followed by the equilibration for 3 min as noted above. Under these conditions the retention times for AMP, ADP and ATP standards used for column calibration were 6.2, 12.3 and 15.9 min, respectively. The concentrations of the nucleotides in neutralized oxygraphic samples were calculated from the peak areas accounting for all dilution factors.
2. Alternatively, the C_{18} reversed phased column (Bondaback 4.5×250 mm) along with the DuPont 8800 HPLC equipment were used. The column

was equilibrated with 100 mM KH_2PO_4 buffer, pH 6.0, and after injection of the sample, eluted with the same buffer solution containing 5% methanol. Elution of the ATP, ADP and AMP standards gave the retention times 9.1, 10.4 and 14.1 min, respectively. Calibrations were performed according to the peak areas of the standard mixtures of low variable amounts of ATP (0–10 μmol) at a constant amount of ADP (100 μmol) and vice versa.

2.5.3. Creatine concentration determination

The total creatine concentration was determined in supernatants after treatment of cardiomyocytes with saponin or Triton X-100 and centrifugation for 5 min at $1000 \times g$. The assay was performed by a colorimetric diacetyl-α-naphtol method after hydrolysis of phosphocreatine in acidic medium and neutralization [18].

2.5.4. LDH determination

The activity of LDH in the supernatants after treatment of cardiomyocytes with saponin or Triton X-100 was assayed as described earlier [19].

2.5.5. Solutions

Solution A contained, in mM: CaK_2EGTA 2.77, K_2EGTA 7.23, $MgCl_2$ 6.56, dithiothreitol (DTT) 0.5, potassium 2-(*N*-morpholino)ethanesulfonate (K-Mes) 50, imidazole 20, taurine 20, Na_2ATP 5.3, phosphocreatine 15, pH 7.1 adjusted at 25°C.

Solution B contained, in mM: CaK_2EGTA 2.77, K_2EGTA 7.23, $MgCl_2$ 1.38, DTT 0.5, K-Mes 100, imidazole 20, taurine 20, K_2HPO_4 3 and pyruvate 5 (or glutamate 5)+malate 2, pH 7.1 adjusted at 25°C.

Solution KCl contained in mM: KCl 125, HEPES 20, glutamate 4, malate 2, Mg-acetate 3, KH_2PO_4 5, EGTA 0.4 and DTT 0.3, pH 7.1 adjusted at 25°C and 2 mg of BSA per ml was added.

2.5.6. Reagents

All reagents were purchased from Sigma (USA) except ATP and ADP which were obtained from Boehringer (Germany).

2.6. Analysis of the experimental results

The values in tables and figures are expressed by means ± S.E.M. The apparent K_m for ADP was estimated from a linear regression of double-reciprocal plots. Statistical comparisons were made using the Anova test (variance analysis and Fisher test), and $P < 0.05$ was taken as the level of significance.

3. Results

3.1. Characteristics of permeabilized muscle fibers and mitochondrial properties inside of them

The permeabilization of the cell membrane by saponin or by digitonin due to their high affinity for cholesterol has very often been used to open the access to intracellular compartments [15]. Table 1 shows that under conditions of such a selective per-

Table 1
Evidence for complete permeabilization of isolated cardiomyocytes by saponin (50 μg/ml, 30 min)

Cell fraction	Total creatine (nmol per mg of protein)	LDH (IU per mg of protein)
1. CMT	69.6 ± 2.1	2.2 ± 0.2
2. CMS	71.8 ± 2.8	2.03 ± 0.15
1. CMST	0.0	0.1 ± 0.04

CMT – supernatant obtained after treatment of cardiomyocytes with 2% Triton X-100 and centrifugation for 5 min at $1000 \times g$. This supernatant contains all cellular pools of metabolites and enzymes (including those in mitochondrial matrix) which are not strongly fixed to myofibrils or cytoskeleton. CMS – supernatant obtained after treatment of cardiomyocytes for 30 min with saponin (50 μg/ml) to selectively permeabilize the sarcolemma, and by subsequent centrifugation for 5 min at $700 \times g$. This fraction contains metabolites and enzymes liberated after permeabilization of sarcolemma and thus localized in the cells in the bulk water phase in the cytoplasm. CMST – supernatant obtained after Triton X-100 (2%) treatment of the pellet obtained after saponin treatment of cardiomyocytes and centrifugation (step CMS). This fraction contains metabolites and enzymes which may be localized in mitochondria or in the cells not permeabilized by saponin (if that was not effective).

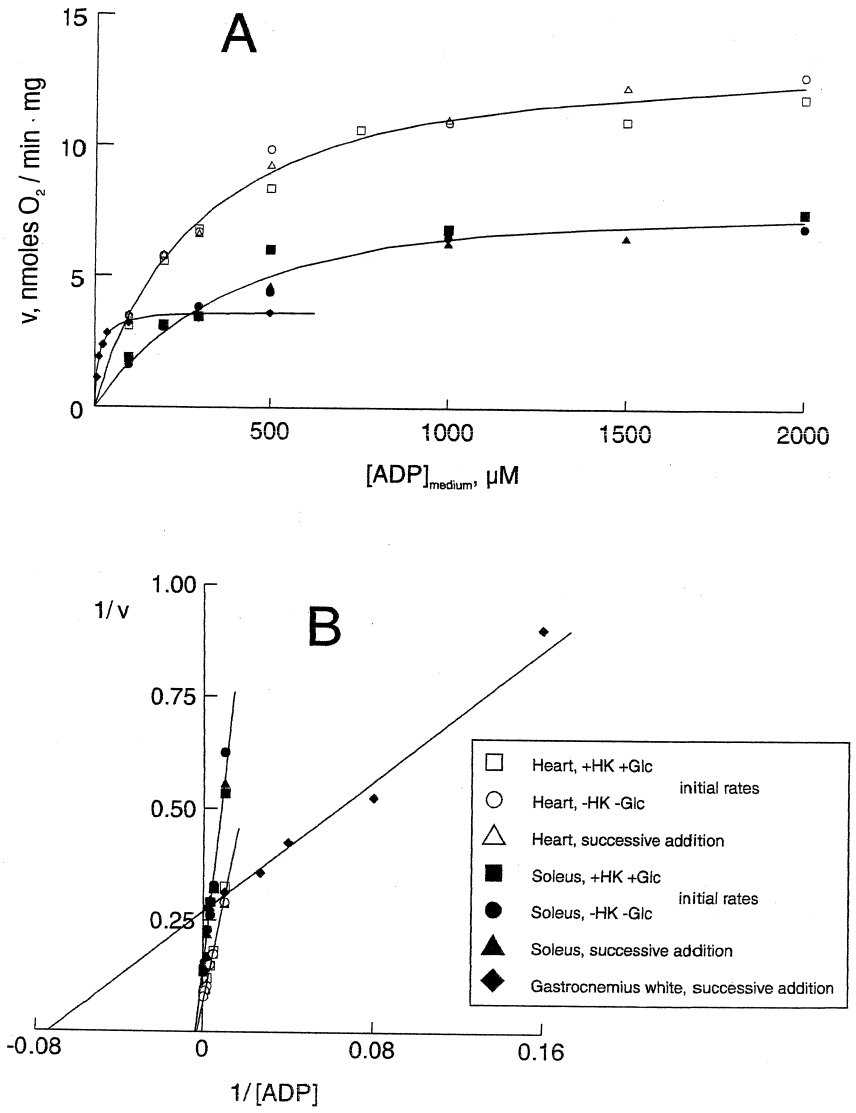

Fig. 1. The kinetics of regulation of mitochondrial respiration in the rat skinned muscle fibers by exogenous ADP. Three different ways of regeneration or addition of exogenous ADP were used, all giving the same results. The initial rates of respiration for each ADP concentration were determined in the presence of the hexokinase–glucose system (open squares for cardiac fibers and closed ones for m. soleus) and in absence of hexokinase–glucose system (open circles for heart fibers and closed ones for m. soleus), or ADP was added successively in the absence of ADP-regenerating hexokinase–glucose system (open triangles for heart and closed triangles for m. soleus fibers). In studies of fibers from m. gastrocnemius white, ADP was added successively in the absence of the hexokinase–glucose system. A: Respiration rates versus ADP concentration in the medium measured by HPLC method. B: Double-reciprocal plot of data from A to determine the apparent K_m values.

meabilization of sarcolemma, all the LDH present in the cells is rapidly released into the medium, and the same is observed for creatine+phosphocreatine pool. In both cases dissolution of mitochondrial membranes by Triton X-100 gives no additional release of neither LDH nor total creatine (Table 1). These results are in good concord with all earlier data of the cytoplasmic localization of LDH, they also show complete permeabilization of sarcolemma by saponin in the concentration used and loss of its barrier function for low molecular weight metabolites and even for macromolecules. At the same time, all functional properties of mitochondria in permeabilized cells are completely intact: the acceptor control ratio (ACR)

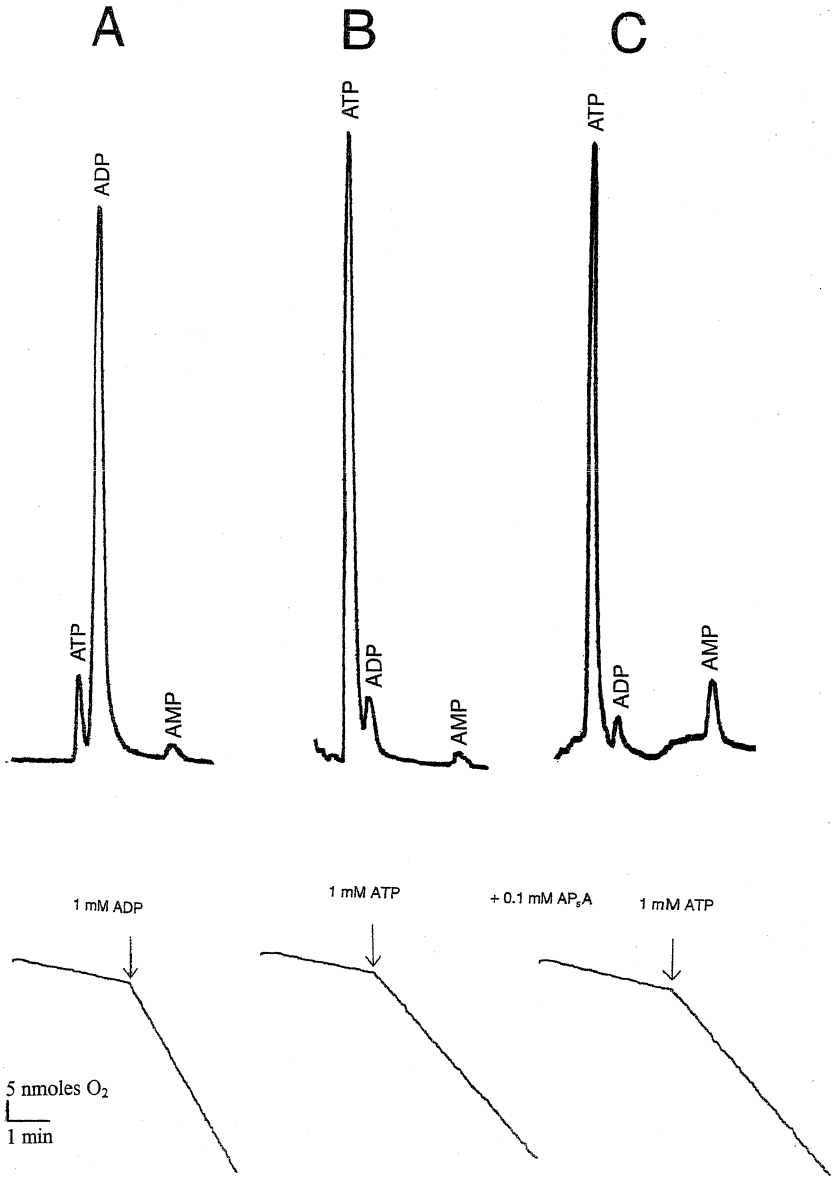

Fig. 2. Mitochondrial respiration in situ when initiated by ADP or ATP. Upper traces are the chromatographic assays of the adenine nucleotides in the medium; lower traces are the oxygraph readings of the respiration rate (recordings of the oxygen concentration change with the time). The samples of the medium were taken for analyses at the end of these recordings, after 3–4 min of the reaction. A: Respiration was initiated by addition of ADP to a final concentration of 1 mM. B: Respiration was initiated by addition of ATP to a final concentration of 1 mM. C: The same as B, but in the presence of AP_5A, 0.1 mM.

of respiration by ADP (2 mM) is close to 10 (in the presence of Mg), demonstrating a high degree of coupling of respiration and oxidative phosphorylation at the inner mitochondrial membrane. Furthermore, in the KCl medium exogenous cytochrome *c* has no effect on respiration rate, demonstrating the intactness and the integrity of outer mitochondrial membrane [10–15] (see also below).

3.2. Kinetics of regulation of mitochondrial respiration by exogenous ADP

The apparent K_m for exogenous ADP was determined with three different protocols to show that the low affinity of mitochondria in situ for exogenous ADP in the medium [10–15] is their intrinsic property. Protocol 1: the initial rate was determined sep-

arately for each ADP concentration assayed directly by HPLC. The exogenous ADP-regenerating system, hexokinase and glucose, was added to avoid any accumulation of ATP. With this technique apparent K_m for exogenous ADP was 300–400 μM in cardiac and m. soleus fibers (Fig. 1). Protocol 2: similar as for protocol 1 but without the added hexokinase+ glucose system. The apparent K_m for ADP was not changed (Fig. 1). Protocol 3: successive additions of ADP in the absence of hexokinase and glucose, when ADP is regenerated solely by endogenous Ca- and Mg-dependent ATPases (Fig. 1). This method requires rapid determinations of respiration rates to avoid significant conversion of ADP into ATP, especially at high rates of oxidative phosphorylation when the activities of the endogenous ATPases are not sufficient for complete regeneration of ADP. (A more precise version of this rapid method could be successive addition of ADP in the presence of hexokinase, 4 IU/ml, and glucose, 11 mM, to ensure complete regeneration of ADP even under conditions of active oxidative phosphorylation.) All these three protocols give the same values of the apparent K_m for exogenous ADP in the medium for cardiac and m. soleus skinned fibers (300–400 μM) and for the m. gastrocnemius white fibers (14 μM) and are in good accordance with all earlier determinations [10–15]. It is interesting to note here that even if there was some accumulation of ATP (up to 20% of added ADP) at high rates of respiration in absence of hexokinase and ADP concentrations were simply calculated from successively added amounts, the apparent K_m value was not changed. An explanation of this paradoxical observation is given below.

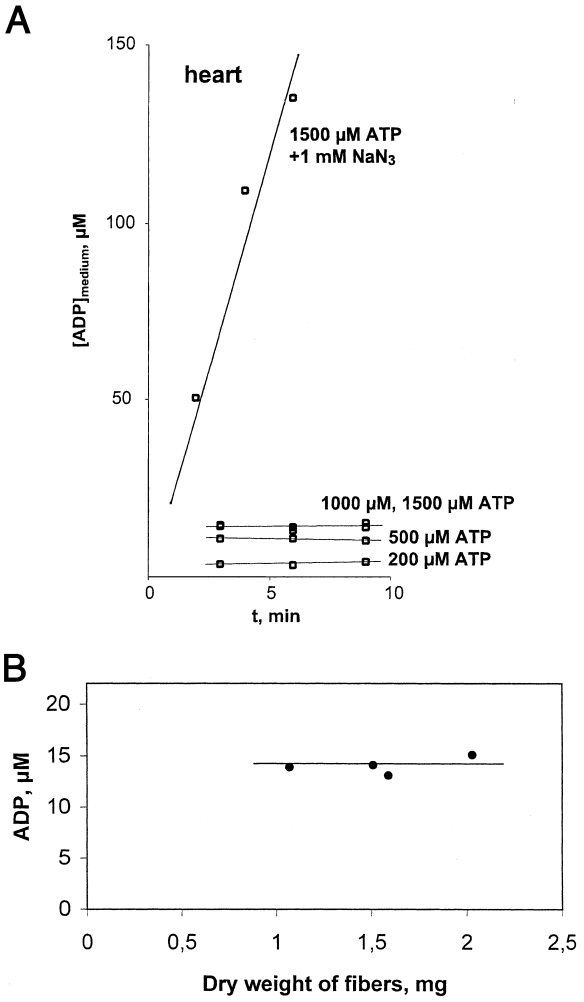

Fig. 3. Changes of ADP concentrations in the medium under conditions of continuous regeneration of endogenous ADP inside the fibers in MgATP reactions. A: Time dependence of ADP concentration in the medium under conditions of oxidative phosphorylation at different ATP concentrations (three lower lines) or in the presence of 1.5 mM ATP when oxidative phosphorylation was inhibited by NaN_3, 1 mM. B: ADP concentration in the medium in the presence of active oxidative phosphorylation activated by 1 mM ATP, at different amounts of fibers added into the oxygraph chamber.

3.3. *Kinetics of regulation of mitochondrial respiration by endogenous ADP generated by intracellular ATPases*

In the permeabilized muscle cells the ATPases in the myofibrils and in the sarcoplasmic reticulum are intact [15]. Therefore, the mitochondrial respiration can be regulated by ADP produced by these ATPases during hydrolysis of exogenous ATP. When 1 mM (submaximal concentration) of exogenous ADP (Fig. 2A) or 1 mM of exogenous ATP (Fig. 2B) are added similar respiration rates are observed. However, despite similar respiration rates the chromatographic analysis of the composition of the reaction medium shows that the concentrations of ADP in the medium are completely different. When exogenous ADP is added, measured concentration is close to that initially added (Fig. 2A, upper trace). On the contrary, when the respiration is started by addition of ATP, high respiration rates are observed at very low ADP concentration in the medium (Fig. 2B, upper trace). In both cases the AMP concentration

in the medium stays very low and constant, and addition of diadenosine pentaphosphate (AP_5A), the inhibitor of the myokinase reaction, has only a minor effect on measured AMP (Fig. 2C). Thus, the myokinase reaction does not contribute to interconversion of adenine nucleotides in any considerable extent under these conditions.

Fig. 3A shows that when oxidative phosphorylation in the cardiac fibers is maintained by endogenous ADP generated in the presence of ATP in different concentrations, the steady state levels of ADP in the medium do not change with time. Fig. 3B shows that under these experimental conditions the steady state levels of ADP in the medium do not depend also on the amount of fibers added into the cell of oxygraph. These data probably mean that the diffusion equilibrium of ADP between bulk water phase inside the fibers and medium and the steady state of reactions involved are established rather rapidly, before the first ADP measurement. Thus, the steady state levels of ADP in medium reflect mainly the relative rates of the MgATPase reactions and oxidative phosphorylation within the fibers. Only if oxidative phosphorylation is inhibited by NaN_3, the ADP concentration in the medium increases with time, as expected, due to the MgATPase activities of fibers. In the latter case, the rate of ADP release is a linear function of the amount of fibers added (data not shown).

Mitochondrial respiration rate shows a hyperbolic relation to exogenous ATP concentration in the medium which in double-reciprocal plots gives a straight line and an apparent K_m value equal to

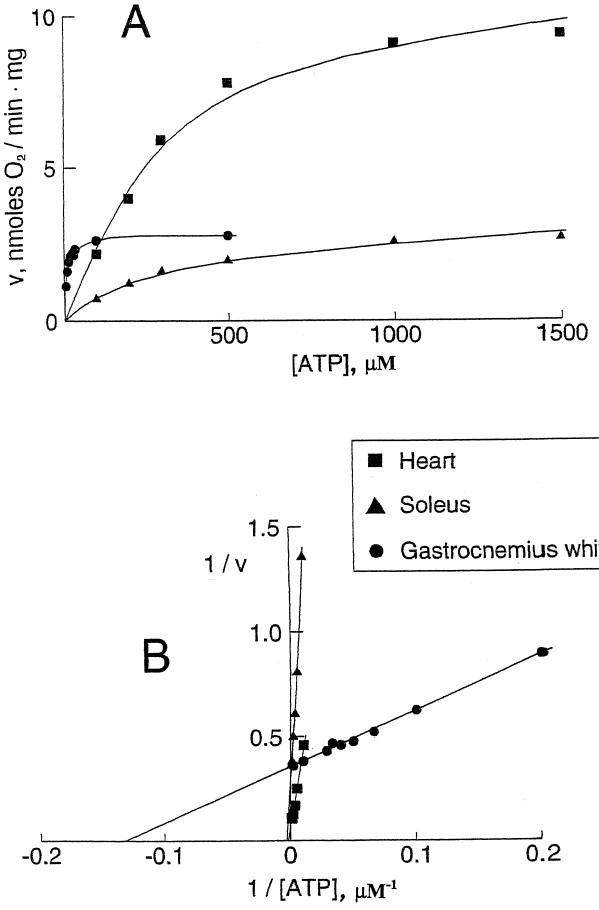

Fig. 4. The apparent kinetics of regulation of mitochondrial respiration by exogenous ATP in the permeabilized fibers from different muscles of rat. The respiration rates of the skinned fibers from heart (squares), m. soleus (triangles) and m. gastrocnemius (circles) were measured after addition of ATP in different final concentrations. A: The respiration rate vs. ATP concentration in a surrounding medium. B: The double-reciprocal plot of data from A.

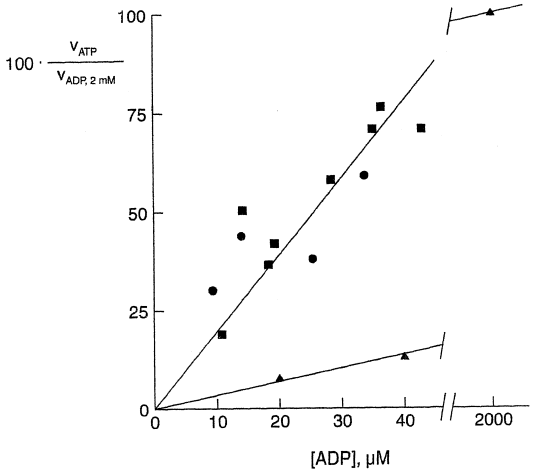

Fig. 5. Dependence of the kinetics of regulation of the rate of mitochondrial respiration by ADP in skinned cardiac fibers in situ upon the source of ADP. The relationships between the ADP concentrations in the assay medium and the respiration rate are shown. The respiration rates are given in relative units, in percents of that observed in the presence of saturating ADP concentration (2 mM). Filled circles (experiments without AP_5A) and squares (experiments in the presence of 0.1 mM AP_5A) show the results of determination of the rates of respiration initiated by addition of different amounts of ATP to regenerate ADP endogenously in the intracellular ATPase reactions. For comparison, the triangles show the kinetics of regulation of respiration by exogenous ADP directly added into medium. In all cases the ADP concentration in the medium which is shown in the abscissa axis was measured by a HPLC method.

Mitochondrial respiration rate shows a hyperbolic relation to exogenous ATP concentration in the medium which in double-reciprocal plots gives a straight line and an apparent K_m value equal to

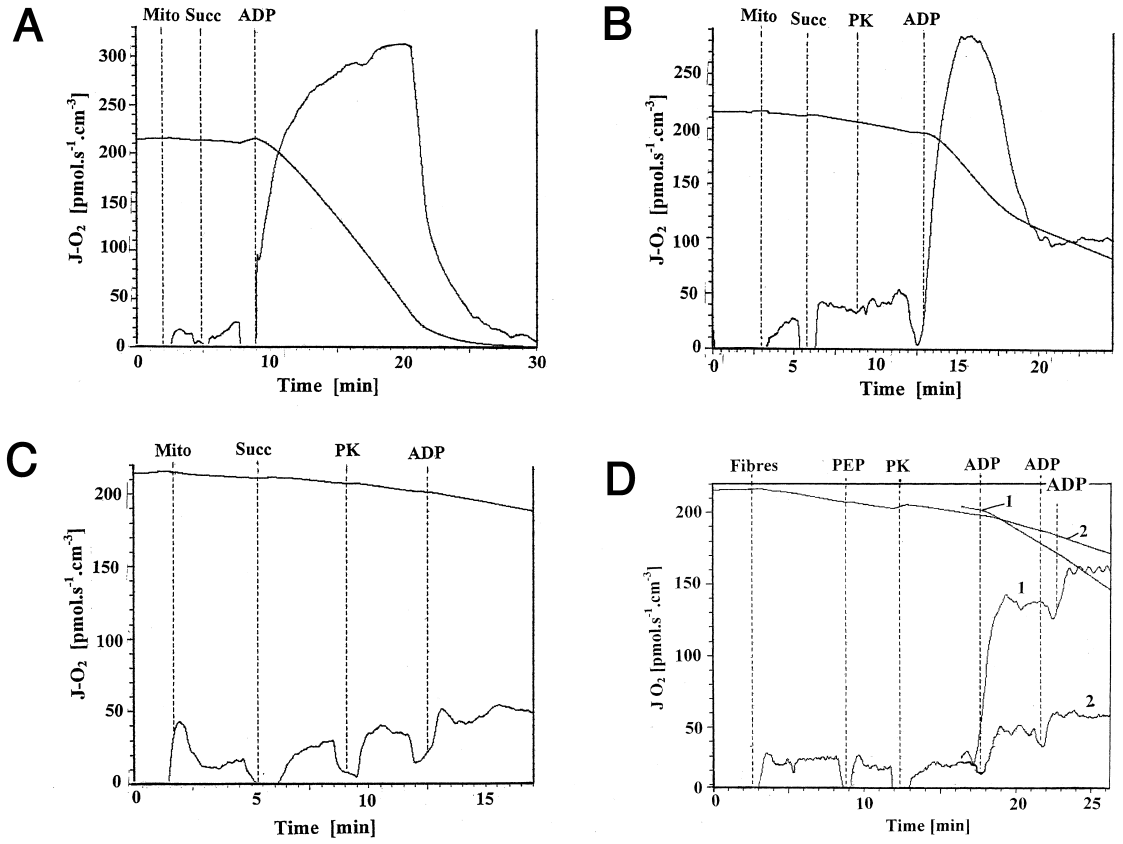

Fig. 6. The effects of PEP+PK system on the respiration of isolated heart mitochondria activated by addition of exogenous ADP (A,B,C) and on the respiration of mitochondria in skinned cardiac fibers in the absence of calcium ions in the sucrose medium (D). All figures show two recordings: the change of oxygen concentration with time (upper curves) and its first derivative (lower curves) showing directly the rate of oxygen consumption. In all experiments described here the sucrose solution (see Section 2) was used. In A, B and C isolated rat heart mitochondria (Mito) were introduced in the respiratory chamber and succinate (Succ), 5 mM, added (state 2). Then, adding of 3 mM of ADP (ADP) induced maximal stimulation of the oxidative phosphorylation. In all cases the reaction medium contained 3 mM PEP. A: Recordings in the absence of PK. B: PK was added in activity of 1 IU/ml before ADP. C: PK was added in activity of 10 IU/ml before ADP. In D, skinned cardiac fibers were used instead of isolated mitochondria. The calcium-free sucrose medium contained glutamate and malate as respiratory substrates. At time-points indicated 0.5 mM and then 1.0 mM exogenous ADP was added. Curves 1 – in the absence of PK+PEP system; curves 2 – PEP (5 mM) and then PK (20 IU) were added before ADP.

300 μM for cardiac and m. soleus fibers (Fig. 4A,B). Again, apparent K_m for ATP in fibers from m. gastrocnemius was much lower, that can be related to the high affinity of mitochondria for ADP in these fibers and higher ATPase activity (see Fig. 1). Thus, the apparent K_m values for both exogenous ADP and exogenous ATP in regulation of mitochondrial respiration are very close and in fact practically the same (compare Figs. 1 and 4). One may conclude from these data that the total concentration of adenine nucleotides, ADP+ATP, is the major determinant of respiration in permeabilized cells. Conversion of added ADP to ATP will therefore have a minor influence on the kinetics and explains why successive addition of ADP in the absence of the added hexokinase system results in a similar high apparent K_m for ADP as the other protocols (see Fig. 1).

The relationship between mitochondrial respiration rate in permeabilized cells and measured ADP concentration in the medium is shown in Fig. 5. When exogenous ATP is added to activate the respiration, the dependence of respiration rate on ADP concentration in the medium is linear up to 40 μM of ADP (Fig. 5). Lower curve in Fig. 5 shows, for comparison, the dependence of the respiration rate on the concentration of exogenous ADP. The slopes of the

linear parts of both curves differ almost by an order of magnitude and demonstrate that ADP generated locally by endogenous ATPases does not equilibrate rapidly with the ADP in the medium.

3.4. The effects of exogenous ADP trapping system: evidence for direct metabolic channeling of ADP

The striking differences in kinetics of regulation of mitochondrial respiration by ADP, in dependence upon the source of ADP described above, show that most probably ADP produced in the intracellular ATPase reactions is directly channeled to mitochondria without significant release into the medium. This hypothesis can be tested by using an exogenous ADP trapping system. In this work we used the PK–PEP system (PK+PEP), which competes with mitochondria for the ADP. This method of competitive enzyme technique has been used previously to identify the preferable metabolic route of ADP in studies of functional coupling of creatine kinase [20,21]. Since the permeabilization of sarcolemma results in rapid release of LDH (Table 1), one may assume that PK is also equally distributed between solution and bulk water phase within the fibers and the reaction medium. First, we tested the efficiency of the PK+PEP system used in experiments with isolated heart mitochondria which have very low MgATPase activity (Fig. 6). Fig. 6A shows that addition of ADP in a concentration of 3 mM activates respiration of isolated rat heart mitochondria (some lag-period in this experiment was due to use of succinate as substrate and activation of succinate dehydrogenase of mitochondria with time, this lag is not seen when glutamate–malate are used as substrates, see below) by a factor close to 8–10, and maintains high respiration rates until the oxygen in the cell is used up. However, if PK was added (PEP was already in the medium) in the final activity of 1 IU/ml before addition of ADP (3 mM), all ADP was used up within 4 min, as it could be predicted from the PK activity, and respiration rate decreased to state 4 level (Fig. 6B). When PK was used in the final activity of 10 IU/ml, all ADP added (3 mM) was used up within seconds and the state 3 of respiration was not observed (Fig. 6C). The PK+PEP system was equally effective in eliminating exogenous ADP when the permeabilized cardiac fibers were used in Ca^{2+}-free sucrose medium to avoid endogenous ADP production in the Ca,MgATPase reactions (Fig. 6D). Activation of mitochondrial respiration in fibers under those conditions by successive addition of 0.5 mM and 1.0 mM exogenous ADP was characterized by ACR ratio 6–7 without PK and PEP. When the PK+PEP was added before ADP, only the state 4 respiration was observed due to very rapid phosphorylation of ADP into ATP (Fig. 6D).

However, very different results were obtained in experiments with permeabilized cardiac fibers in the solution B which contained both Mg^{2+} and Ca^{2+} ions and therefore Ca,MgATPases were activated in the fiber cells. In these experiments the activity of added PK (20 IU/ml) exceeded the combined rates of the ATPase and oxidative phosphorylation in oxygraphic cells (maximally 0.2 μmol per min per ml) by two orders of magnitude. The ACR of respiration for saturating concentrations of ADP (2 mM), in the presence of Mg^{2+}, was close to 10 (Fig. 7A), very close to that in experiments with isolated mitochondria (Fig. 6A). Addition of exogenous ATP in final concentration of 2 mM activates respiration about 4–5 times and addition of ADP (2 mM) activates respiration further to the maximal rate (ACR about 8–10, Fig. 7B). Despite the presence of a powerful ADP trapping system (PK+PEP) the rate of respiration initiated by addition of 2 mM ATP decreased only about 20–30% (Fig. 7C). Further addition of PK had no effect on the respiration rate. Our earlier calculations and experiments with soluble ADP-regenerating systems have shown that in the homogenous system when intermediate diffusion is not limited, 20-fold excess of PK over the competing ADP-consuming process completely inhibits the latter [20,21]. Thus, the results shown in Fig. 7C demonstrate that endogenously generated ADP is not easily accessible for PK if mitochondrial respiration is activated: instead, the latter process has a preferable access to endogenous ADP. The respiration rate showed a further decrease after addition of AP_5A, but even then the respiration rate stayed above the v_0 level (Fig. 7C). When PK+PEP were added before addition of exogenous ADP (2 mM), the latter was instantly converted into ATP as indicated by low ACR = 4 (ACR was 4, Fig. 7D – note that in the presence of exogenous ADP it is 8–10, see above) and demonstrates that the PK+PEP system used

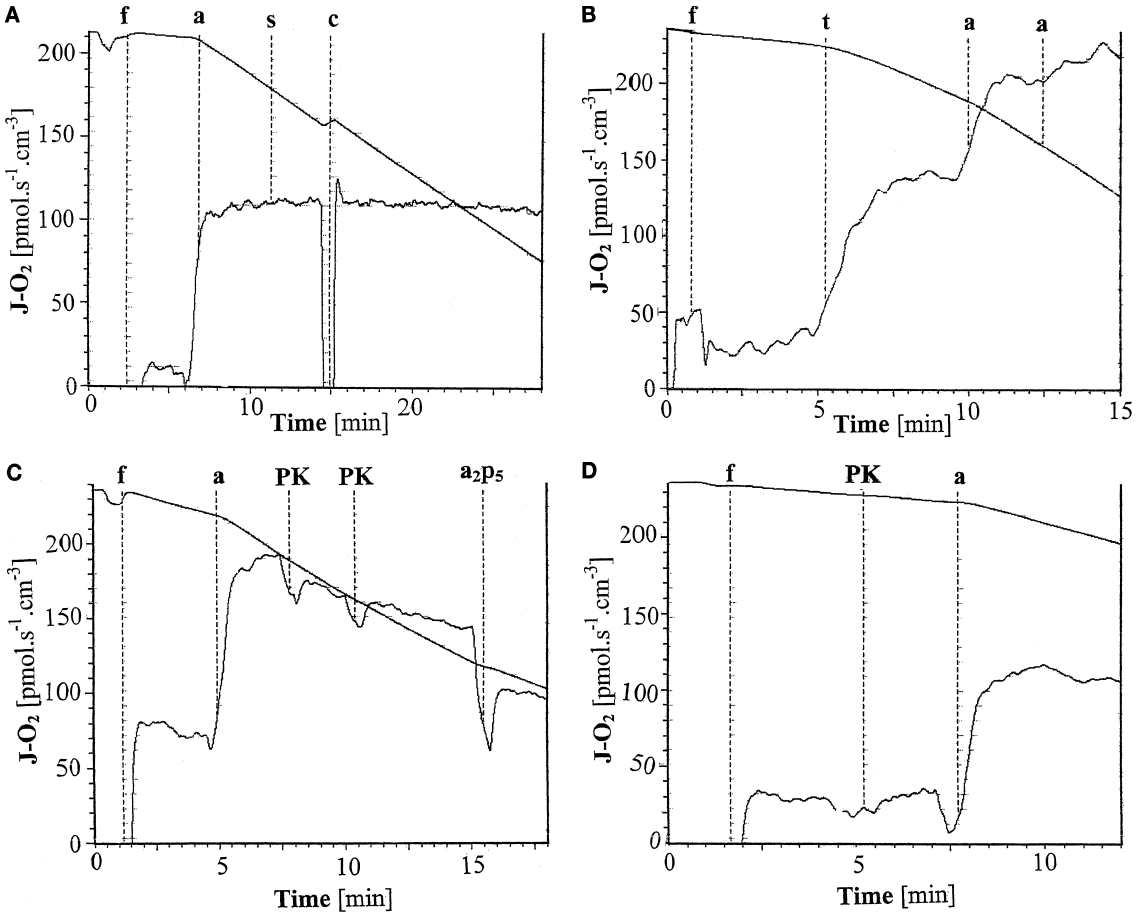

Fig. 7. Oxygraphic recordings of the effect of the exogenous ADP trapping system of PK and PEP on the respiration of skinned cardiac fibers induced by exogenous ATP or ADP. A: Respiratory parameters of the skinned rat cardiac fibers. Upper trace – oxygen concentration changes in time; lower trace – the first derivative of the upper trace, the rate of respiration. Additions: fibers-permeabilized fibers; ADP – 2 mM; succinate – 2 mM; cytochrome *c* – 8 μM. The reaction rates were measured in KCl medium (see Section 2). The ratio of respiration rates after and before ADP addition is called ACR and is close to 10. Absence of any effect of cytochrome *c* addition shows perfect preservation of the outer mitochondrial membrane (see [13]). B: First ATP, 2 mM, and then ADP, twice 1 mM, in total 2 mM were added. ATP gave only about 70% of V_{max} of respiration. Note that the final value of ACR after addition of ADP was close to 8. These and all further measurements were made in solution B with 4 mM $MgCl_2$ (see Section 2). C: The respiration of skinned fibers was initiated by addition of ATP, 2 mM, in the presence of PEP (5 mM) in the medium. Then PK, 20 IU, was added twice and finally AP_5A (0.1 mM) was added. D: The medium contained 5 mM PEP and 10 IU/ml of PK. Respiration was started with addition of 2 mM ADP, but ACR 3 shows that ADP was instantly converted into ATP (see B and C).

was very powerful in removing ADP from medium, as verified in experiments described in Fig. 6. Earlier we have found that in the presence of PK+PEP and AP_5A the ADP concentration in the medium, determined by HPLC, was less than 10–14 μM [22]. Thus, the endogenously produced ADP was not fully accessible to the powerful exogenous competitive enzymatic system, but still available to mitochondria. That means that ADP was directly channeled from the endogenous ATPases to mitochondria.

The ADP flux through PK+PEP system was measured directly by a spectrophotometric method using LDH and NADH (Fig. 8). In this coupled system, the rate of oxidation of NADH is equivalent to the rate of ADP release into the medium (Fig. 8A). Addition of exogenous ATP resulted in a decrease in NADH with the stable steady state rate corresponding to a release of ADP (Fig. 8B). When the mitochondrial respiratory substrates glutamate and malate were added the rate of NADH oxidation decreased by about 50% (Fig. 8B,C) and when mitochondrial uptake of ADP was inhibited by atractylo-

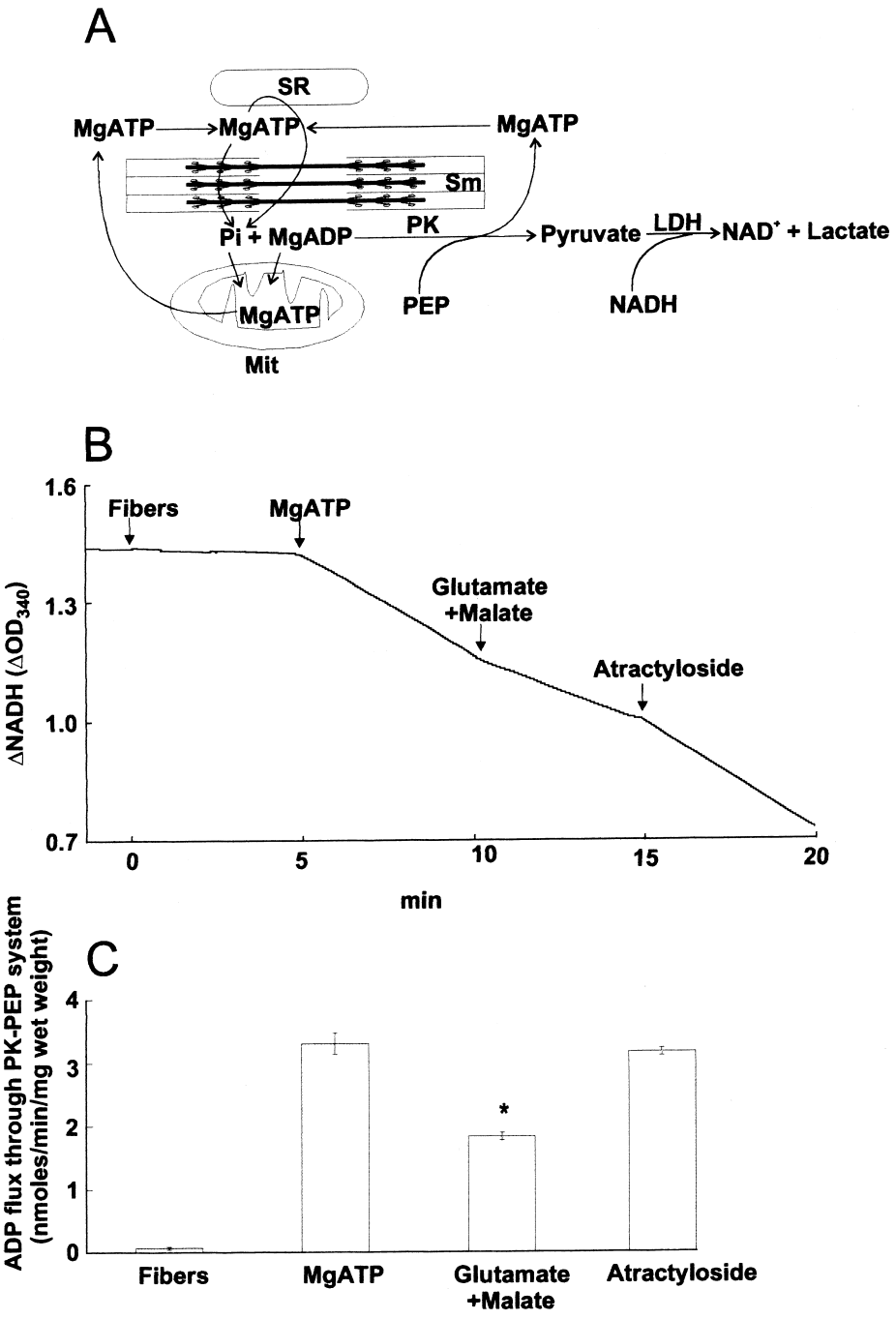

Fig. 8. Changes of the rate of release of ADP from cardiac muscle fibers and its flux through the PK+PEP system by activating or inhibiting the mitochondrial oxidative phosphorylation. A: A scheme of the ATPase reactions coupled either to mitochondrial oxidative phosphorylation or to extramitochondrially added PK–PEP system of ADP rephosphorylation. B: The original recordings of the changes of the ADP flux through PEP–PK system, estimated as the decreased NADH content in the reaction medium, prior and after subsequent additions of ATP (1 mM), substrates (10 mM glutamate and 5 mM malate) and atractyloside (98 μM). C: The means ± S.E.M. for three experiments shown in B. $*P < 0.01$ compared to flux prior to addition of glutamate and lactate.

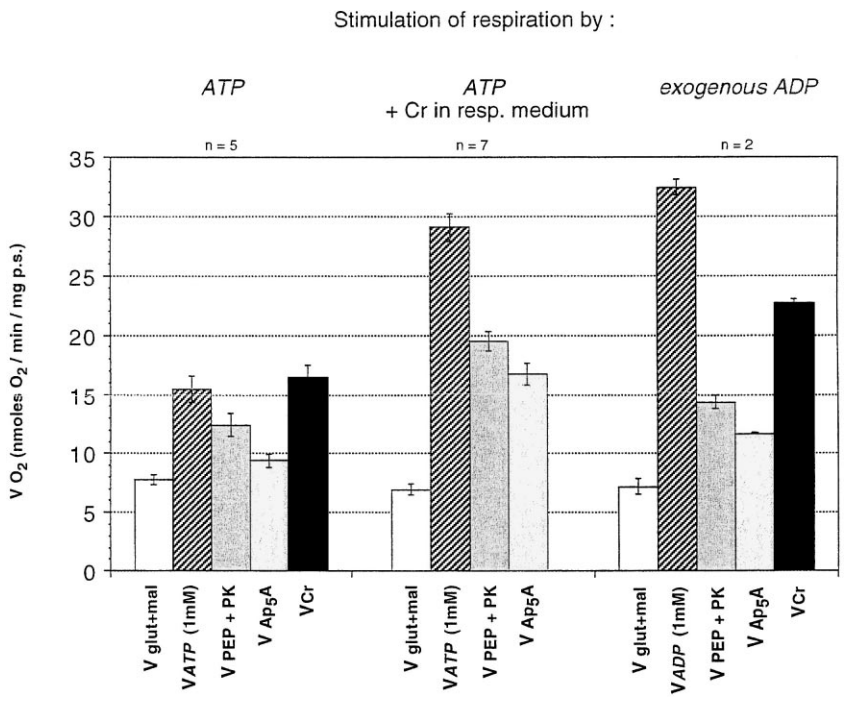

Fig. 9. Stimulation of the mitochondrial respiration by creatine in the skinned fibers from mice heart in the presence of ATP or ADP with PK+PEP. The reaction medium contained 5 mM PEP. Additions: fibers-skinned cardiac fibers from mice; ATP – 1 mM; PK – 20 IU/ml; Cr – creatine, 20 mM. Statistic treatment of the experimental data, the average values and S.E.M. are shown for groups of 2–7 experiments. For explanation see the text.

side, the rate of oxidation of NADH was restored to its initial value (Fig. 8B,C). These results give further support for the hypothesis that ADP generated by intracellular MgATPases is first available for mitochondria, before its release into the medium.

3.5. The effects of creatine and AP_5A

If ADP is channeled directly from ATPases to mitochondria, the interesting question that immediately follows is that of the role of the creatine kinase system which has been classically considered as an energy transfer system ('shuttle', 'circuit') between mitochondria and energy utilizing systems [12,23,24]. Fig. 9 shows that the role of the creatine kinase system is still there: addition of creatine (20 mM) in the presence of 1 mM ATP and exogenous PK+PEP system gives strong increase in the respiration rate (Fig. 9). In our recent study we have shown that these stimulating effects of creatine are observed at very low constant ADP concentrations in the medium and not observed if the mitochondrial creatine kinase is knocked out by genetic manipulations [22]. The importance of the creatine kinase system in the energy transfer function between intracellular sites of free energy utilization and production is evident.

4. Discussion

The results of this study show that in permeabilized oxidative muscle the kinetics of regulation of mitochondrial respiration by exogenous ADP are very different from the kinetics of its regulation by endogenous ADP. This may be taken to show that in these cells mitochondria, consuming ADP and producing ATP, behave as if they were included into functional complexes with adjacent ADP producing systems – with the MgATPases of myofibrils and Ca,MgATPases of sarcoplasmic reticulum. ADP produced within these units, or complexes, does not equilibrate easily with ADP in the bulk phase. In the case of permeabilized cells the bulk phase is a medium, in the cells in vivo – it is probably a cytosol.

Moreover, within these functional units the energy is transferred mostly via enzyme networks composed of the creatine kinase and adenylate kinase systems.

An important property of the isolated cardiomyocytes or permeabilized muscle fibers used in this work is that all soluble cytosolic enzymes and metabolites, the markers of the bulk water phase of the cell, are completely released into the medium. Further, if ADP is freely diffusing within the bulk water phase within fibers as usually accepted [25], calculations by using the diffusion coefficient for ADP in water solutions [25] and Einstein–Smoluchowski equation for Brownian movement show that the time of displacement of ADP from the center of cardiomyocytes into the medium is in the range of tens of milliseconds and thus the diffusion of ADP out of intracellular bulk water phase into medium is very rapid [26]. This conclusion was recently confirmed by mathematical modelling, using the reaction–diffusion model of energy transfer [27] (see also below). Thus, one may assume that there is rapid diffusion equilibrium of metabolites including ADP and ATP between bulk water phase in fibers and medium. Nevertheless, the relationship between ADP concentration in the medium and respiration rate of mitochondria in situ in skinned fibers is highly dependent upon the source of ADP. This phenomenon was in general terms described first by Kummel [28] who, similar to the present results, observed high values for an apparent K_m for exogenous ADP in regulation of respiration in permeabilized isolated cardiomyocytes. The observed apparent K_m exceeds the value of this parameter for mitochondria in vitro by an order of magnitude [10–15]. Previous studies have documented that the presence of creatine, due to continuous regeneration of the local ADP behind the outer mitochondrial membrane, in the intermembrane space of mitochondria in the coupled mitochondrial creatine kinase reaction, decreases the apparent K_m for exogenous ADP several times [10–15]. Similar decrease of apparent K_m for exogenous ADP in regulation of mitochondrial respiration in vivo was achieved by short rather selective proteolytic treatment of permeabilized cells or by rupture of outer membrane after hypo-osmotic treatment of fibers [10–15]. Two conclusions were made from these observations. First, it was concluded that the ADP diffusion may be restricted at the level of outer mitochondrial membrane due to the control of porin pores in these membranes by some cytoplasmic proteins [11–13]. This is in concord with many observations by Colombini et al., who showed that the porin channel permeability may be easily regulated and it may become practically impermeable for adenine nucleotides [29,30]. Since high apparent K_m for exogenous ADP was not dependent on the rate of oxidative phosphorylation–ADP consumption [15], not changed after extraction of myosin (still high in 'ghost' fibers) but very sensitive to proteolytic treatment [11,13], and not observed in glycolytic fibers in spite of their larger diameter [17], the observed phenomena could not be explained by simple diffusion restrictions of ADP in myofibrillar space as it was initially proposed [10]. However, even more relevant to the results reported here may by the second conclusion. It was concluded that the high apparent K_m for exogenous ADP seems to show the existence of the structurally organized feedback signaling system in the cells [13] which operates by the vectorial ligand conduction mechanism, as originally proposed in general terms by Peter Mitchell [31]. Due to such a structural organization, the mitochondria become not easily accessible for exogenous ADP, and endogenously generated ADP does not equilibrate easily with the ADP in the medium. There are two lines of direct experimental evidence for these pathways for endogenous ADP. Firstly, the observation that mitochondrial respiration was similar despite striking difference in concentration of ADP in the medium when ADP was generated exogenously or endogenously (see Fig. 2). The second line of evidence is presented by the results of experiments with the competing PK+PEP system (Figs. 7–9). The latter show that most of the endogenously generated ADP is not easily accessible for the exogenous PK and PEP, if the mitochondrial oxidative phosphorylation is actively running. Since exogenous PK can occupy the intracellular bulk water phase from where the LDH has been released during permeabilization, these results mean that the endogenously generated ADP is not released into this bulk water phase.

This conclusion is in analogy with the recent results of confocal microscopic studies of calcium release from sarcoplasmic reticulum. By using fluorescent calcium probes and calcium-sensitive photoprotein targeted to the outer phase of the inner mi-

tochondrial membrane it was directly demonstrated that the release of calcium from sarcoplasmic reticulum was focal and occurred in the vicinity of mitochondria [32–35]. Duchen et al. proposed that sarcoplasmic reticulum and mitochondria 'may be intimately associated as a functional unit' [33]. The results of this work show that not only calcium but also ADP may be focally released from the MgATPases of reticulum and myofibrils into the vicinity of mitochondria. The results described are consistent with the view that in oxidative muscle cells mitochondria may be associated into functional units not only with sarcoplasmic reticulum but also sarcomeres of myofibrils and that ADP released focally into the space inside these units does not equilibrate rapidly with the ADP in the medium. Recent analysis of ADP profiles in myofibrillar space by reaction–diffusion mathematical model showed that if the diffusion constant for ADP was taken to be equal to that in the water, its increase by several orders of magnitude did not change the metabolic profiles between mitochondria and myofibrils [27]. That again means that in the bulk water phase the ADP diffusion is sufficiently rapid and allows equilibration of ADP concentration in the intracellular bulk water phase and in the medium, as discussed above. However, decrease of the diffusion constant value (restriction of ADP diffusion) in the model strongly influenced the ADP profiles inside the myofibrillar space (inside the functional units described) [27]. There may be significant restriction of ADP diffusion within these units at the level of mitochondrial outer membrane (control of VDAC, see above) and/or in myofibrils, since our experiments (see Fig. 9) show directly that both ADP diffusion and energy transfer are facilitated by activation of the energy transfer network, the creatine kinase system [14]. These results are also consistent with the conclusion of Moerland's group [36] that the sarcoplasmic reticulum and mitochondria may be the principal intracellular structures that decrease the diffusional mobility of phosphocreatine in an orientation-dependent manner (anisotropy).

Neely and Grotyohann and others have shown that about 70% of total cellular ATP can be removed from the cell (by hypoxic degradation or by perfusion with deoxyglucose) without alteration of the contractile function of the heart provided that the creatine kinase system stays intact [37]. Thus, we may assume that about 30% of the cellular pool of adenine nucleotides is actively involved in the metabolic channeling pathways within functional units discussed above.

The differences between the metabolites and enzymes in the bulk phase of intracellular water and that inside the organized metabolic systems and multienzyme complexes observed in this work support the theoretical concepts of cell architecture and metabolic channeling developed by the groups of Srere, Ovadi and Clegg [38–40]. This concept of eukaryotic cell organization considers that virtually all the cell architecture is interconnected (by the 'microtrabecular lattice' containing cytoskeletal elements), with the intervening aqueous phase(s) being extremely dilute with respect to dissolved macromolecules [39,40]. At the same time, there is no sharp, rigid discontinuity between the 'architecture' and the surrounding 'aqueous phase' [40]. Klopfenstein et al. showed recently that direct interaction of endoplasmic reticulum with microtubules is mediated by an integral protein p63 [41], and multiple connections between mitochondria and cytoskeleton elements have been described in the muscle and other types of cells ([16,42], also reviewed in [43,44]). Therefore, the structural organization of these functional complexes is probably also related to the organization of the cytoskeletal network of the cell. However, this very important question requires further very careful study.

Acknowledgements

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